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Jha, Awadhesh



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# Evaluation of interactive effects of phosphorus-32 and copper on marine and freshwater bivalve molluscs

Emily L. Vernon[a], Michael N. Moore[abc], Tim P. Bean[de] and Awadhesh N. Jha[a*]

[a]School of Biological and Marine Sciences, University of Plymouth, Plymouth, PL4 8AA, UK

[b]European Centre for Environment & Human Health (ECEHH), University of Exeter Medical School, Truro, TR1 3HD, UK

[c]Plymouth Marine Laboratory, Plymouth, PL4 3DH, UK

[d]Cefas Weymouth Laboratory, Barrack Road, The Nothe, Weymouth, Dorset DT4 8UB, UK

[e]Present address: The Roslin Institute, The University of Edinburgh, Easter Bush Campus, Midlothian, EH25 9RG

*Correspondence: a.jha@plymouth.ac.uk

***Running Title: Effects of radionuclide and metal on mussels***

## Abstract

**Purpose:** Contaminants seldom occur in isolation in the aquatic environment. While pollution of coastal and inland water bodies has received considerable attention to date, there is limited information on potential interactive effects between radionuclides and metals. Whether by accidental or controlled release, such contaminants co-exist in aquatic ecosystems and can pose an enhanced threat to biota. Using a range of biological responses, the study aimed to evaluate relative interactive effects on representative freshwater and marine bivalve species.

**Methods:** An integrated, multi-biomarker approach was adopted to investigate response to copper (Cu, 18 μg $L^{-1}$), a known environmentally relevant genotoxic metal and differing concentrations of phosphorus-32 ($^{32}P$; 0.1 and 1 mGy $d^{-1}$), alone and in combination in marine (*Mytilus galloprovincialis*) and freshwater (*Dreissena polymorpha*) mussels. Genetic and molecular biomarkers were determined post-exposure and included DNA damage (as measured by the comet assay), micronuclei (MN) formation, γ-H2AX foci induction and the expression of key stress-related genes (i.e. *hsp70/90, sod, cat, gst*).

**Results:** Overall, using a tissue-specific (i.e. gill and digestive gland) approach, genotoxic response was reflective of exposures where Cu had a slight additive effect on $^{32}P$-induced damage across the species (but not all), cell types and dose rates. Multivariate analysis found significant correlations between comet and γ-H2AX assays, across both the tissues. Transcriptional expression of selected genes were generally unaltered in response to contaminant exposures, independent of species or tissues.

**Conclusions:** Our study is the first to explore the interactive effects of ionizing radiation (IR) and Cu on two bivalve species representing two ecological habitats. The complexity of IR-metal interactions demonstrate that extrapolation of findings obtained from single stressor studies into field conditions could be misrepresentative of real-world environments. In turn, environmental protective strategies deemed suitable in protecting biota from a single, isolated stressor may not be wholly adequate.

**Keywords:** Bivalve, $^{32}$Phosphorus ($^{32}$P), Genotoxicity, Gene expression, Metal

## Highlights

- Adoption of an integrated, multi-biomarker approach in two bivalve species
- Toxicity of combined mixtures of $^{32}$P and Cu compared
- Cu induced additive effects with $^{32}$P in the tissues
- DNA damage and DDR showed strong correlations
- Multiple stressors should be considered in assessing the impact of ionizing radiations

**Abbreviations:** µg, microgram; γ-H2AX, Gamma Histone 2AX; $^{60}$Co, Cobalt 60; $^{137}$Cs, Caesium 137; $^{3}$H, Tritium; $^{32}$P, Phosphorus 32; $^{32}$S, Sulphur 32; $^{90}$Sr, Strontium 90; ACT, Actin; ATP, Adenosine triphosphate; Bq, Becquerel; BHA, Butylated hydroxyanisole; CAT, Catalase; cDna, Complimentary DNA; CPM, Counts per minute; Cq, Threshold cycle; Cu, Copper; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; DDR, DNA damage response; DG, Digestive gland; DI, Deionised water; DO, Dissolved oxygen; DOC, Dissolved organic carbon; DP, *Dreissena polymorpha*; DSB, Double strand break; EF1, Elongation factor 1; ERICA, Environmental Risk from Ionizing Contaminants: Assessment and Management; GST, Glutathione-S-Transferase; HCl, Hydrochloric acid; HSP, Heat Shock Protein; ICP-MS, Inductively coupled plasma mass spectrometry; IR, Ionizing radiation; LET, Linear energy transfer;

LMA, Low melting point agarose; LSC, Liquid Scintillation Counting; MBq, Mega Becquerel; MG, *Mytilus galloprovincialis*; mGy, Milligray; MN, Micronuclei; MOA, Mechanism of action; MT-10, Metallothionein 10; NPP, Nuclear power plant; PCA, Principal component analysis; PCR, Polymerase chain reaction; qPCR, Quantitative PCR; RER, Relative expression ratio; ROS, Reactive oxygen species; SOD, Superoxide Dismutase; SSB, Single strand break; TBHQ, Tertiary-butyl hydroquinone

## 1 Introduction

Radionuclides and metals co-exist and are often co-discharged in the aquatic environment. Their increased prominence results primarily from anthropogenic activities (e.g. industrial discharge, nuclear power generation and decommissioning, accidents or weapons tests, mining, wastewater treatment; Salbu et al. 2005; Hu et al. 2010; Skipperud and Salbu 2018). Nuclear power plants (NPPs), as an example co-dispose radionuclides and non-radioactive wastes. The requirement for large volumes of water for cooling processes typically results in NPPs being located close to large water bodies (i.e. coasts, lakes), where waste products are disposed under set regulations. Whilst discharges of contaminants are largely controlled, little is known about potential interactive effects (Pearson et al., 2018). Exposure to a mixture of stressors which in isolation may not induce significant damage may cause deleterious effects on organism's health through additive or synergistic mechanisms when acting in combination (Eggen et al. 2004; Salbu et al. 2005; Mothersill et al. 2007; Olsvik et al. 2010; Heier et al. 2013; Dallas et al. 2012, 2016).

It is well accepted that radionuclides and metals can readily bioaccumulate and concentrate in the tissues of aquatic organisms, posing a threat to both humans via food consumption and biota by trophic level transfer (Carvalho 2018). When present within a cell, such contaminants have varying potential to cause significant damage to molecules such as DNA, both directly (direct interaction with DNA molecules or other cellular structures) or indirectly (generation of reactive oxidative species [ROS]), the extent dependent on concentration, linear energy transfer (LET), distribution and biological half-life. Short-lived

radionuclide phosphorus-32 ($^{32}P$, half-life = 14.29 d) was utilised in this study as a cost-effective, accessible surrogate for more environmentally ubiquitous beta and gamma emitting radionuclides (e.g. caesium-137; $^{137}Cs$, strontium 90; $^{90}Sr$). In comparison to other radionuclides there is limited information regarding its environmental presence. $^{32}P$ reference levels, referring to concentrations that, when consumed at 2 L $day^{-1}$ equate to a total ingested dose of 0.10 mSv $y^{-1}$ (in humans) are fixed at 57 Bq $L^{-1}$ (DWQR, 2014). Environmental background levels average 0.27 ± 0.21 Bq $L^{-1}$ (2005-2013, River Clyde, Erskine Harbour, King George V Dock, SEPA 2013). Despite its short half-life it could readily be accumulated by organisms and concentrated in tissues, particularly following chronic exposures. In terms of biological impact, $^{32}P$ is chemically and radiologically distinct (Cheng et al. 2015). Subsequent to the incorporation of aqueous $^{32}P$ into the ribose-phosphate backbone of replicating DNA, isotopic decay ($^{32}P$ to sulfur-32, $^{32}S$) results in chemical breakage of DNA (single strand breaks, SSBs). More detrimental DNA damage (double strand breaks, DSBs) are induced via the release of high-energy beta particles. As demonstrated by us previously, $^{32}P$ readily accumulated in the tissues of mussels and detrimental $^{32}P$-induced responses (DNA damage, micronuclei [MN] and γ-H2AX foci induction) have been noted at dose rates as low as 0.1 mGy $d^{-1}$ (Vernon et al. 2018; 2020). Similarly, radionuclides such as $^{137}Cs$ and tritium ($^{3}H$) have been found to induce biological damage across multiple aquatic species including molluscs (*C. gigas*, *Mytilus spp*.), arthropods (*D. magna*) and fish (*D. rerio*, *O. mykiss*, *O. latipes*) (Hagger et al. 2005; Jha et al. 2005; Jha et al. 2006; Jaeschke et al. 2011; Pereira et al. 2011; Devos et al. 2015; Parisot et al. 2015; Dallas et al. 2016; Sayed et al. 2017; Pearson et al. 2018; Festarini et al. 2019).

For example at low $^{3}H$ doses (~15.58 µGy $h^{-1}$), Dallas et al. (2016) noted upregulation of genes involved in protein folding, DNA DSB repair and cell cycle checkpoint control in *M. galloprovincialis*, transcriptome level effects were well correlated with genetic (DNA) damage.

Metals such as copper (Cu) can be highly toxic to organisms at concentrations present within marine and freshwater environments. Although biologically essential, playing an important role in numerous physiological and biochemical processes (i.e. growth, metabolism and enzymatic activities), Cu ions are redox-active and facilitate the production of ROS, leading to oxidative damage to biomolecules including DNA, proteins and lipids (Cid et al. 1995; Gaetke and Chow 2003; Bopp et al. 2008; Kim et al. 2008). Typically, permitted Cu levels in England and Wales are 5 µg $L^{-1}$ in seawater and between 1–28 µg $L^{-1}$ in freshwater (dependant on water hardness, DEFRA 2014), however total dissolved Cu in contaminated environments, such as around NPPs can reach concentrations of 689 µg $L^{-1}$ (Bryan and Gibbs 1983). Cu-induced damaging effects in aquatic invertebrates are well studied (Al-Subiai et al. 2011; Vosloo et al. 2012; Brooks et al. 2015; Xu et al. 2016; Vernon and Jha, 2019), and much research has focused on interactions between Cu and additional stressors such as metals, ocean acidification, temperature, pesticides, microplastics (Clayton et al. 2000; Bouskill et al. 2006; Trevisan et al. 2011; Maria et al. 2013; Lewis et al. 2016). However, to our knowledge no studies have determined the potential interactive effects of IR and Cu in mussels.

In this study the effects of $^{32}P$ and Cu, alone and in combination were studied in two ecologically relevant adult bivalve species. *Mytilus galloprovincialis* (MG) and *Dreissena polymorpha* (DP) were selected as representatives of marine

and freshwater environments (McDonald et al. 1991; Binelli et al. 2015; Vernon et al. 2018; Vernon and Jha, 2019). Mussels are well established model species in ecotoxicological studies and as such the physiology, anatomy and ecology of MG and DP are well understood. This multi-species exposure enables determination of relative sensitivity and is arguably a more robust, environmentally representative approach than single-species exposures (Chapman 2002; Solomon and Sibley 2002; Schnug et al. 2014).

A suite of sub-lethal biological responses or biomarkers were measured in the digestive gland (DG) and gill cells of the selected mussel species, following exposure to a range of $^{32}P$ and Cu, alone and in combination. $^{32}P$ dose rates of 0.10 and 1 mGy $d^{-1}$ (Table 1) were in line with a generic screening value (all species) of 10 µGy $h^{-1}$ (0.24 mGy $d^{-1}$), where no significant negative effects are expected at the population level (Andersson et al. 2008; Andersson et al. 2009). Cu concentrations (18 µg $L^{-1}$) were reflective of environmental realistic values, and in line with previous studies (Al-Subiai et al. 2011; Vernon and Jha 2019). The overall aims and objectives of this study were (a) to adopt an integrated, multi-biomarker approach in investigating combined effects of Cu and $^{32}P$ in two bivalve species, (b) to determine potential correlations between different parameters (molecular, genetic) studied and (c) to determine relative sensitivity between different cell types (i.e. gill and digestive gland cells). Principal Component Analysis (PCA) combined with Hierarchical Cluster Analysis was utilised to integrate data from molecular and genetic biomarkers. With regards to species variation, we hypothesised firstly that little disparity in response will be evident. Secondly, Cu would have a modifying (i.e. additive or synergistic) effect on the $^{32}P$-induced biological responses in mussels.

**Table 1.** Table to show the expected dose rates in mGy $d^{-1}$ and the average dose rate achieved in *M. galloprovincialis* and *D. polmorpha* whole-body, digestive gland and gill tissue (mGy $d^{-1}$). . Values in bold are those above the ERICA tool screening value of 0.24 mGy $d^{-1}$.

| | | Av. Dose rate (mGy $d^{-1}$) | | |
|---|---|---|---|---|
| | Expected dose rate | Whole body | Digestive gland | Gill |
| *M. galloprovincialis* | 0.1 | 0.11 | **4.34** | 0.09 |
| | 1 | **0.96** | **38.76** | **0.66** |
| *D. polymorpha* | 0.1 | 0.08 | **1.53** | **0.8** |
| | 1 | **0.87** | **3.72** | **1.68** |

## 2 Materials and methods

### 2.1.1 Chemicals and suppliers

Radiolabelled-ATP (Adenosine triphosphate, $\gamma$-$^{32}$P) was purchased from Perkin Elmer (PerkinElmer, UK, 9.25 MBq: specific activity: 370 MBq $mL^{-1}$) and diluted with DI water to form appropriate working stocks (Vernon et al. 2018). All working solutions were decay adjusted throughout the exposure. Where appropriate, product details for chemicals and reagents are noted within the text.

### 2.1.2 Mussel exposure conditions

Mussels were collected from a reference site and maintained as described in detail in other studies from our research group (Dallas et al. 2013; Dallas et al. 2016; Pearson et al. 2018; Vernon et al. 2018).

Ten-day exposures were performed between September-October 2017. MG and DP experiments were staggered by two weeks for logistical reasons. Following collection and after a 2-week acclimation, MG (mean wet weight: 12.17 ± 0.67 g) and DP (mean wet weight: 3.22 ± 0.27 g) individuals (total wet weight, 35 g $L^{-1}$) per labelled beaker (9 mussels $treatment^{-1}$; 3 mussels $beaker^{-1}$) were exposed to the following exposure scenarios in triplicate: (a) 0.1 mGy $d^{-1}$, (b) 0.1 mGy $d^{-1}$ + Cu, (c) 1 mGy $d^{-1}$ and (d) 1 mGy $d^{-1}$ + Cu. The Cu (CuSO4.5H2O, 99% purity) concentration used in combination with $^{32}P$ was 18 µg $L^{-1}$. Control and positive control ($CuSO_4.5H_2O$, 56 µg $L^{-1}$) treatments were run alongside. $^{32}P$ activity levels in water were calculated in a previous study (Vernon et al. 2018). To meet expected nominal dose rates $^{32}P$ activity concentrations for 0.1 mGy $d^{-1}$ were 993 Bq $L^{-1}$ (MG) and 579 Bq $L^{-1}$ (DP), and for 1 mGy $d^{-1}$, 9930 Bq $L^{-1}$ (MG) and 5786 Bq $L^{-1}$ (DP).

Mussels were fed (2 h before 50 % water change) on days 3, 5, 7 and 9, as described in earlier studies (Vernon et al. 2018). Water quality parameters (pH, temperature, salinity and dissolved oxygen [DO]) were measured before and after water changes. $^{32}P$ and Cu activity levels were determined using water samples (1 mL, in duplicate), taken ~30 min after each water change and processed for liquid scintillation counting (LSC) or ICP-MS, as described in section 2.3. LSC counting and ICP-MS data confirmed that achieved values across all treatment groups were in line with nominal concentrations (Table 2).

**Table 2.** Water quality parameters (pH, temperature, salinity and dissolved oxygen [DO]), copper concentrations in water (µg $L^{-1}$) and $^{32}P$ concentrations in water (Bq $L^{-1}$), for both species. Data is presented as mean ± standard deviation. *n* = 3.

| **Water parameters** | ***M. galloprovincialis*** | ***D. polymorpha*** |
|---|---|---|
| pH | 8.1 ± 0.06 | 8.1 ± 0.08 |
| Temp (°C) | 14.6 ± 0.24 | 14.7 ± 0.23 |
| Salinity | 36.7 ± 3.90 | 0.3 ± 0.03 |
| DO (%) | 99.4 ± 2.15 | 93.2 ± 1.33 |
| **Copper water conc. (µg $L^{-1}$)** | | |
| Control | 1.9 ± 0.08 | 0.3 ± 0.02 |
| 0.1 mGy $d^{-1}$ + Cu | 14.0 ± 0.72 | 17.1 ± 0.21 |
| 0.1 mGy $d^{-1}$ | 2.4 ± 0.24 | 0.3 ± 0.07 |
| 1 mGy $d^{-1}$ + Cu | 15.8 ± 0.49 | 17 ± 0.14 |
| 1 mGy $d^{-1}$ | 4.1 ± 0.56 | 0.4 ± 0.16 |
| 56 µg $L^{-1}$ | 40.9 ± 2.09 | 56.0 ± 0.60 |
| **$^{32}P$ water conc. (Bq $L^{-1}$)** | | |
| Control | 0.8 ± 1.04 | 0.2 ± 0.58 |
| 0.1 mGy $d^{-1}$ - 993 / 579 | 1246.3 ± 360.94 | 573.2 ± 444.04 |
| 1 mGy $d^{-1}$ - 9930 / 5786 | 9712.8 ± 1235.16 | 4641.0 ± 2128.19 |

### 2.1.3 Sampling procedures

After exposures, gill and digestive gland tissues were dissected from each individual, other soft tissue and shell was discarded. $^{2}/_{3}$ tissue was stored in

tube (on ice, 4 ºC) until isolation of cells and $^1/_3$ in RNAlater (1.5 mL, Fisher UK) at -20 ºC.

## 2.2 Biological assays

### 2.2.1 Isolation of digestive gland and gill cells

Gill and digestive gland cells were extracted in accordance with Vincent-Hubert et al. (2011), with minor adjustments. Samples were incubated with 1 mL dispase II solution (37 ºC, 30 min) and shaken every 10 min. Following incubation, cell suspension was centrifuged (775 g, 5 min) and supernatant utilised in consequent assays. Viability of cells was greater than (<) 90% across all treatments, as determined using the Trypan Blue exclusion dye assay (Strober 2001, data not included).

### 2.2.2 Comet assay to determine DNA strand breaks, analysis of micronuclei (MN) and γ-H2AX foci induction

Biological assays are described in detail in previous studies from our laboratory (Jha et al. 2005; Cheung et al. 2006; Al-Subiai et al. 2011; Dallas et al. 2013; Pearson et al. 2018; Vernon et al. 2020). DNA strand breakage was determined using the single-cell gel-electrophoresis or comet assay. Comet IV imaging software (Perceptive Imaging, Bury St Edmunds, UK) was used for scoring the cells (100 cells per slide). Genotoxicity data are presented as % Tail DNA in accordance to previous studies (Kumaravel and Jha 2006; Vernon et al. 2020). MN induction and γ-H2AX expression analysis are described in full in Vernon et al. (2020). MN were scored following widely accepted criteria (Venier et al.

1997; Bolognesi and Fenech 2012). The results were reported as mean MN per 1000 cells in accordance with previously published data from our laboratory (Dallas et al. 2013). In terms of γ-H2AX expression analysis, number of γ-H2AX foci in each of 50 cells per individual/slide were counted using a fluorescence microscope (NIKON Epifluorescence 80i, 60x magnification). An appropriate DAPI filter was used to locate cell nuclei and a FITC filter set for the FITC signal of the primary antibody (anti-GamaH2H, Mouse Monoclonal Antibody, Sigma) for γ-H2AX foci (Festarini et al. 2015).

### 2.2.3 Determination of transcriptional expression of key genes

RNA extraction, cDNA synthesis and Real-time Polymerase chain reaction (qPCR) were performed in accordance to Vernon et al. (2020).

**Table 3.** Gene and primers used for *M. galloprovincialis* and *D. polymorpha* in RT-PCR.

| Gene | Short name | Forward primer | Reverse primer | GenBank Accession No. |
|---|---|---|---|---|
| | | *Mytilus galloprovincialis* | | |
| Actin | *act* | 5' -GGGAGTCATGGTTGGTATGG- 3' | 5' -TCAGAAGGACTGGGTGCTCT- 3' | AF157491 |
| Elongation factor 1 | *ef1* | 5' -CACCACGAGTCTCTCCCAGA- 3' | 5' -GCTGTCACCACAGACCATTCC- 3' | AF063420 |
| Glutathione S-transferase | *GST* | 5' -ATGGCTCTTTTCCTGCTTCA- 3' | 5' -TTTGGCCAGTGTCCATGTTA- 3' | AF527010 |
| Superoxide dismutase | *CuZn-sod* | 5′ -TGCAGGATCACATTTCAACCCA-3′ | 5′ -TGTCTTGCTTAGCTCATGGCCA-3′ | AJ581746 |
| Catalase | *CAT* | 5' -CACCAGGTGTCCTTCCTGTT- 3' | 5' -CTTCCGAGATGGCGTTGTAT- 3' | AY743716 |
| Heat-shock Protein 70 | *HSP70* | 5' -GGGTGGTGGAACTTTTGATG- 3' | 5' -GCCGTTGAAAAAGTCCTGAA- 3' | AF172607 |
| Heat-shock Protein 90 | *HSP90* | 5' -TCAGTGATGATCCTAGATTAGGCA- 3' | 5' -CGTTCCTCTCTTTCCATCTGTAAC- 3' | AJ625655 |
| | | *Dreissena polymorpha* | | |
| Actin | *act* | 5' -CCTCACCCTCAAGTACCCCAT- 3' | 5' -TTGGCCTTTGGGTTGAGTG- 3' | AF082863 |
| Elongation factor 1 | *ef1* | 5' -CCACCAAAGGCAGCCAAGAG- 3' | 5' -TGGGACGAGGTCAGCCATAC- 3' | AJ250733 |
| Glutathione S-transferase | *GST* | 5' -TCCGCTATATCTGCCTGGAC- 3' | 5' -GCTCCTTCAGACCTGCTTTC- 3' | EF194203 |
| Superoxide dismutase | *SOD* | 5' -GACAGCATGGCTTCCATGTG- 3' | 5' -AGATTCTGGGCCAGTCAGAG- 3' | AY377970 |
| Catalase | *CAT* | 5' -ACGGCTATGGAAGCCACACG- 3' | 5' -AGGTCGCGCATCGCATAGTC- 3' | EF681763 |
| Heat-shock Protein 70 | *HSP70* | 5' -TGTCCTGCTTGTGGATGTAG- 3' | 5' -CGTGGTGAATGTCTGTGTAG- 3' | EF526096 |
| Heat-shock Protein 90 | *HSP90* | 5' -TTGATCATCAATACTTTCTATTC- 3' | 5' -ACACCAAACTGTCCAATCAT- 3' | GU433881a |

## 2.3 Water quality measurements and $^{32}$P and Cu analyses

### 2.3.1 $^{32}$P activity level analysis in water samples

Prior to liquid scintillation counting (Hidex 300SL), water samples were combined with 4 mL ScintLogic U scintillant (LabLogic Systems Ltd, UK) in 20 mL borosilicate glass (Fisherbrand™) vials. Samples, including blanks were stored in dark (~ 2) and read for 10 sec (in triplicate). Samples were decay adjusted (due to short half-life of $^{32}$P) and background corrected by subtracting blanks. An activity of 0.000 was assigned to CPM (counts per min) sample values below the blank (Vernon et al., 2018; Jaeschke and Bradshaw, 2013).

### 2.3.2 Cu concentration analysis in water samples

Immediately post extraction, water samples were spiked with 50 µl HCl, and stored until analysis (room temperature). In line with Dallas et al. (2013), indium (115-In) and iridium (193-Ir) were added as internal standards. In accordance with Al-Subiai et al. (2011), appropriate Cu standards were used to calibrate the instrument before and during analysis of samples. Following dilution of seawater samples (1:5, DI water), standards, samples and blanks (run every 10 samples) were analysed using X Series II ICP-MS (Plasma Quad PQ2 Turbo, Thermo Elemental, Winsford, UK) with PQ Vision 4.1.2 software.

## 2.4 Dosimetry and the ERICA TOOL

The ERICA (Environmental Risk from Ionizing Contaminants: Assessment and Management) tool was used for dose estimation, as described in detail in

Vernon et al. (2018, 2020). Dose rates for whole-body and tissue-specific $^{32}P$ concentrations are presented in Table 1.

### 2.5 Statistical analysis

LinRegPCR (version 11, Ramakers et al. 2003; Ruijter et al. 2009) and threshold cycle (Cq) was utilised to determine PCR efficiencies. From such values, the relative mRNA expression ratio (RER) of the 5 selected genes was quantified (REST software [v 2009, Qiagen Ltd], Dallas et al. 2013; Dallas et al. 2016), relative to reference genes actin (*act*) and elongation factor 1 (*ef1*), using control values as calibrators.

Statistics were performed in R (RStudio, R 3.4.3 GUI 1.70 El Capitan build (7463), https://www.r-project.org/). Where applicable, data were checked for normality and homogeneity of variance, with visual inspection of residuals. Appropriate non-parametric (Kruskal-Wallis with Dunn's pairwise comparison with Bonferroni correction) or parametric tests (one-way ANOVA was run with Tukey's post hoc, with Wilcoxon rank sum test with Holm-Bonferroni correction). Significance level was fixed at $p < 0.05$ (*) and data (unless noted otherwise) shown as mean ± standard deviation.

#### 2.5.1 Multivariate analysis

Biomarker data for gill and digestive gland tissues in both species were analysed using multivariate analysis software, PRIMER v 6.1.5 (PRIMER-Є Ltd., U. Auckland, New Zealand; Clarke 1999, Clarke and Warwick 2001), in accordance with Sforzini et al. (2020). Possible 'significant biomarkers', those

which capture the full PCA biological response pattern were identified using PRIMER v6 - BIO-ENV routine.

## 3 Results

Cu and $^{32}P$ concentrations, along with water quality measurements are displayed in Table 2. Whole body and specific tissue (i.e. gill and digestive gland) dose rates are illustrated in Table 1. Whole body dose rates were 0.11 and 0.96 for MG and 0.08, 0.87 mGy $d^{-1}$ for DP, in line with predicted values.

### 3.1 Genotoxic response following *in vivo* exposures to $^{32}P$ and Cu

Fig. 1 shows mean (A) % tail DNA damage, (B) MN per 1000 cells and (C) γ-H2AX induction in MG and DP gill and digestive gland cells, following exposure to varying concentrations of $^{32}P$ and Cu, alone and in combination for 10 days. Potential correlations within the biomarker data are displayed on Figs. 2a and b, A – D. Control (unexposed) treatments show a low degree of damage across all biomarkers, indicative of good health in both mussel species.

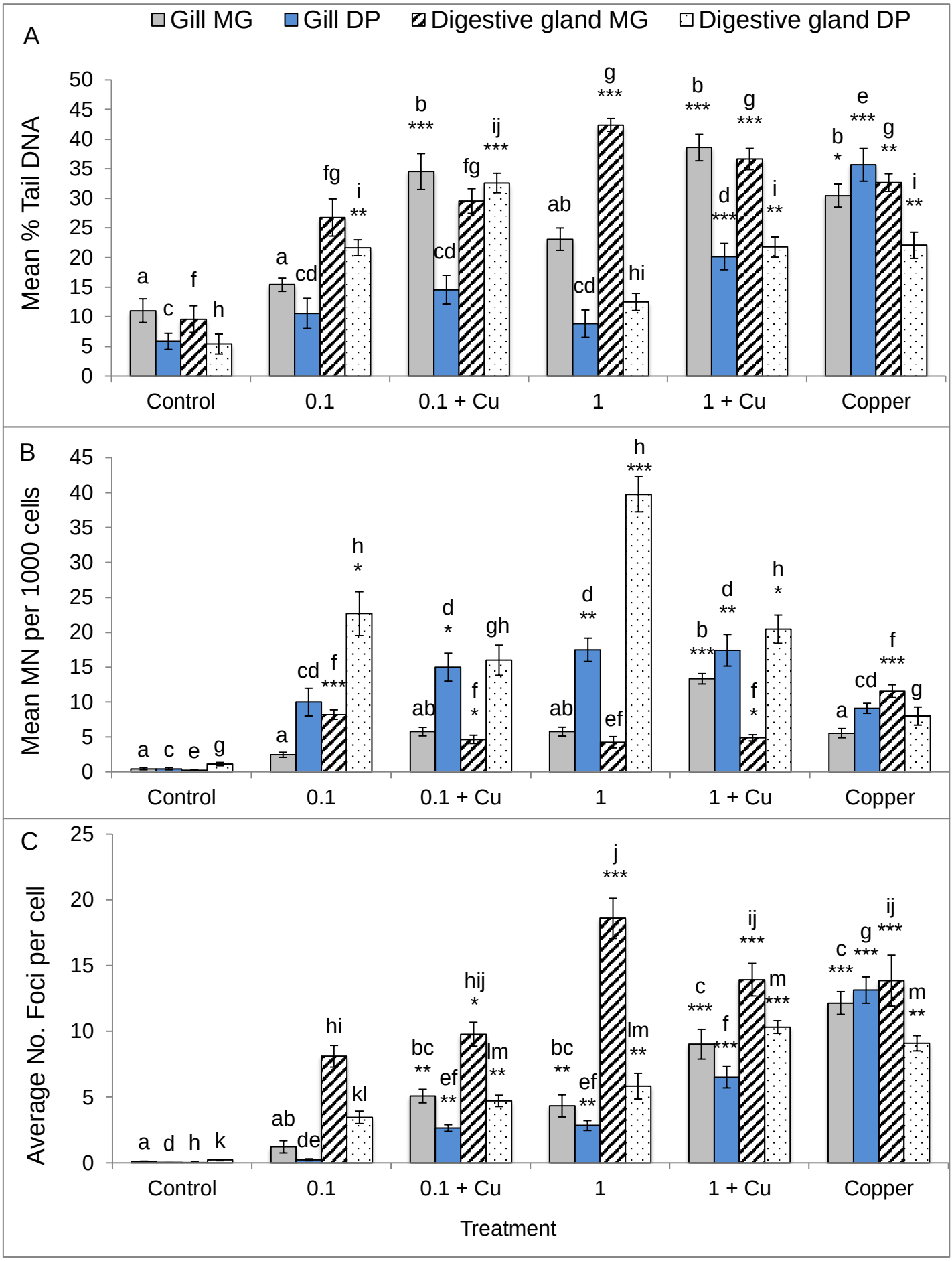


**Figure 1.** Genotoxic effects and subsequent repair in *M. galloprovincialis* and *D. polymorpha* gill and digestive gland cells following a 10 day exposure to $^{32}P$ and Cu, alone and in combination. Asterisks (*, ** or ***) are indicative of significant differences ($p < 0.05$, 0.01, 0.001) from the corresponding control. Letters are indicative of significant differences ($p < 0.05$) between species tissue (i.e. MG gill tissue). SD is standard deviation of mean data, $n = 9$. Copper = positive control.

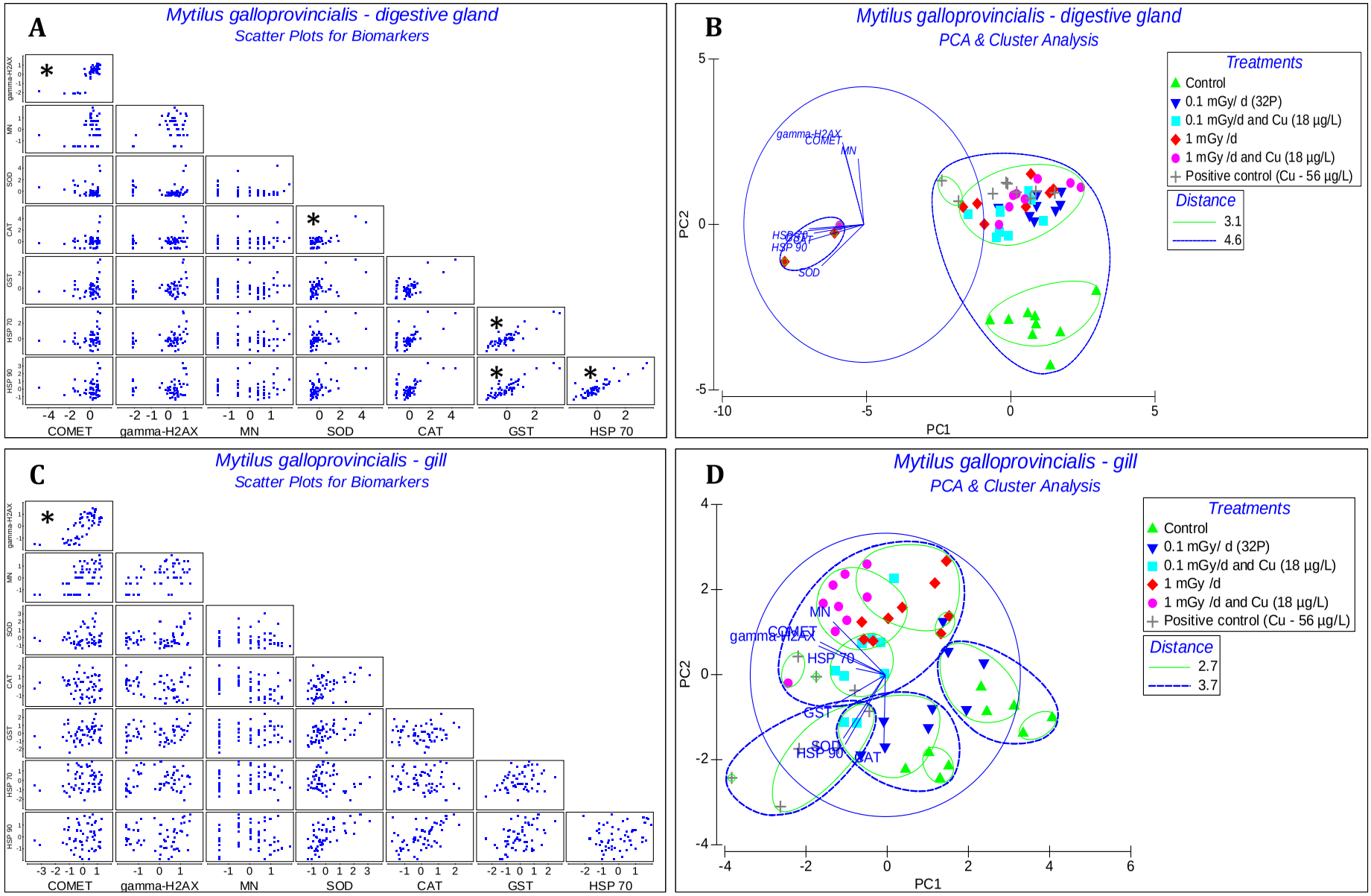


**Figure 2a**. Multivariate analysis (PRIMER 6.1.5) between treatments for the integrated biomarker data showing scatter plots (A, C), principal component (PCA) and cluster analysis of the biomarker data. Vectors indicate the directionality of specific biomarkers. (A, B) *M. galloprovincialis* digestive gland and (C, D) gill tissue. Asterisks (*) are indicative of significant differences ($p < 0.05$). PC1 captured (B) 47.5 % and (D) 32.9 % of the variation.

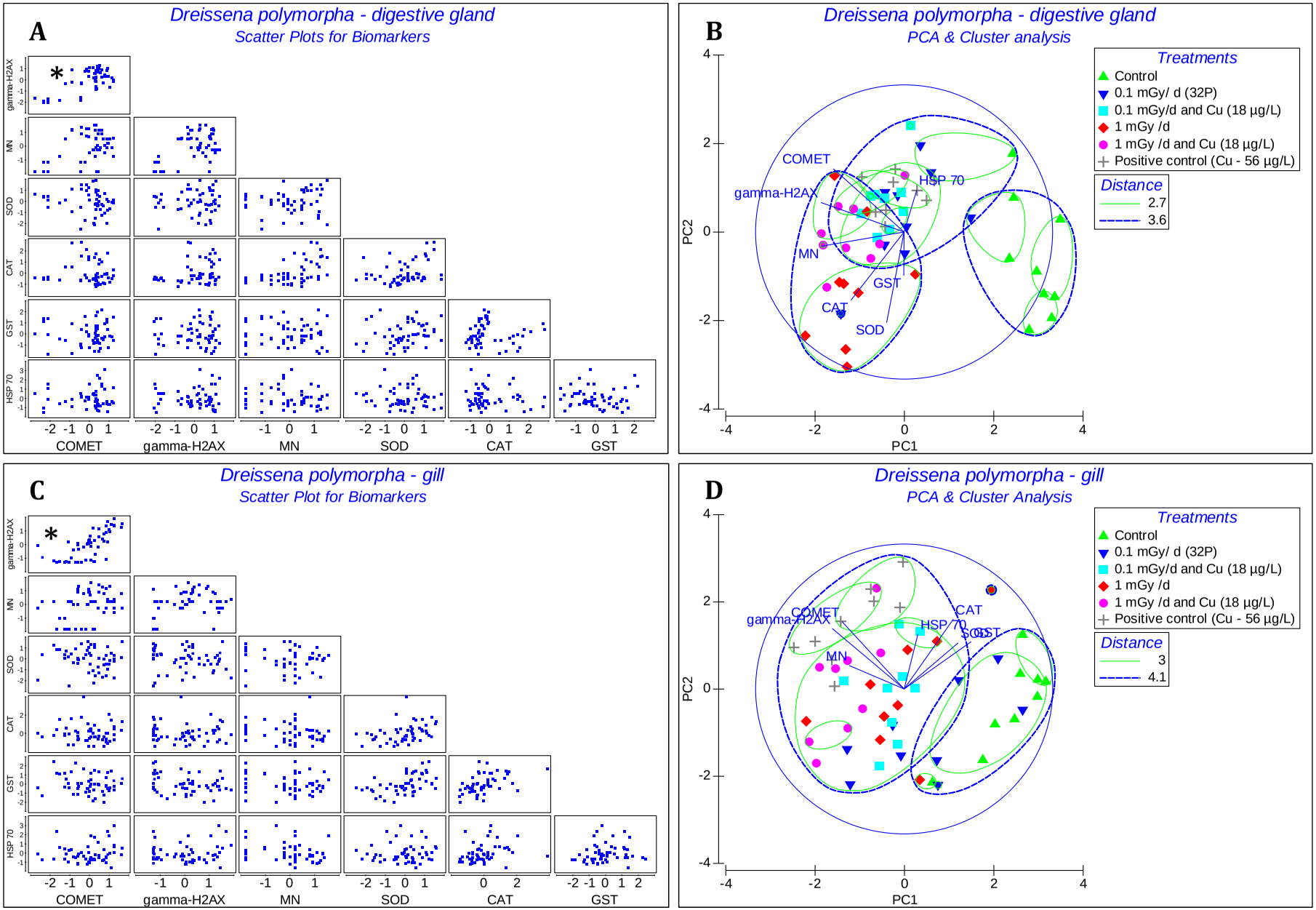


**Figure 2b**. Multivariate analysis (PRIMER 6.1.5) between treatments for the integrated biomarker data showing scatter plots (A, C), principal component (PCA) and cluster analysis of the biomarker data. Vectors indicate the directionality of specific biomarkers. (A, B) *D. polymorpha* digestive gland and (C, D) gill tissue. Asterisks (*) are indicative of significant differences ($p < 0.05$). PC1 captured (B) 32.6 % and (D) 33.2 % of the variation.

### 3.1.1 Comet assay to determine DNA strand breaks

While the only significant ($p < 0.01$) interaction between stressors was evident in MG gill, between the 0.1 and 0.1 mGy $d^{-1}$+ Cu treatments, Cu had a weak interaction with $^{32}P$ across all treatments excluding MG digestive gland (1 and 1 mGy $d^{-1}$+ Cu), where the addition of Cu decreased the damaging effect of $^{32}P$ (Fig. 1A). The lowest degree of damage was evident in DP gill tissue across all treatments, % Tail DNA was only significantly greater than controls with the addition of Cu (1 mGy $d^{-1}$ + Cu, $p < 0.001$). This trend was continued in MG gill (both treatments). Cu appeared to have the greatest interaction with $^{32}P$ within this tissue, where % Tail DNA was increased by 2.3- and 1.7-fold (0.1 and 1 mGy $d^{-1}$, respectively).

### 3.1.2 Analysis of micronuclei (MN) formation

Trends observed in DNA damage response were not reflected for MN formation (Fig. 1B), as Cu did not appear to interact with $^{32}P$ in a detrimental manner (excluding MG gill). No significant difference was noted between treatments in DP gill, MG and DP digestive gland. The greatest degree of damage was evident in DP digestive gland, particularly in the 1 mGy $d^{-1}$ treatment at 40 MN/1000 cells. Cu appeared to have an antagonistic interaction with $^{32}P$, where MN induction decreased when exposed to both stressors. In terms of relative sensitivity DP shows a higher MN frequency in gill and digestive gland cells over its marine counterpart, across all treatments (excluding negative/positive controls). However only significantly so between gill cells in the 1 mGy $d^{-1}$ treatment ($p < 0.05$).

### 3.1.3 Induction of γ-H2AX foci

Fig 1C shows the average number of γ-H2AX foci per cell, indicative of DSBs. In keeping with DNA damage, an enhanced number of γ-H2AX foci were evident in MG digestive gland, particularly in 1 mGy $d^{-1}$ treatment at 19 foci/cell. On average, γ-H2AX foci in MG digestive gland was 2.3– to 3.1- fold higher than DP. In relation to the corresponding control, no significant increase in γ-H2AX foci/cell were noted at the lowest $^{32}P$ treatment of 0.1 mGy $d^{-1}$ across both species and tissues, however, foci number significantly increased with the addition of Cu (MG digestive gland: $p < 0.05$, MG and DP gill, DP digestive gland: $p < 0.01$).

### 3.2 Transcriptional expression of key genes

Due to failure of the assay to amplify, *hsp90* data are not included for DP. For key genes studied in DP, PCR efficiencies were (primer details, table 3): *actin* (*act*): 1.80, *elongation factor 1* (*ef1*): 1.79, *catalase* (*cat*): 1.82, *glutathione-s-transferase* (*gst*): 1.79, *superoxide dismutase* (*sod*): 1.78 and *heat shock protein 70* (*hsp70*): 1.81. In MG: *act:* 1.79, *ef1:* 1.79, *cat:* 1.81, *gst:* 1.81, *sod:* 1.80, *hsp70:* 1.75 and *heat shock protein 90* (*hsp90*): 1.83. Relative gene expression of key genes is presented in figure 3. Overall there was limited variation across all biological tissue and species, with no change evident in MG digestive gland. At 1 mGy $d^{-1}$, *cat* was downregulated in MG gill, but upregulated in DP digestive gland ($p < 0.001$). Downregulation of *cat* was further noted in MG gill 1 + Cu treatment, but to a lesser extent ($p < 0.01$). In response to Cu, *sod* was significantly upregulated in MG gill ($p < 0.01$).

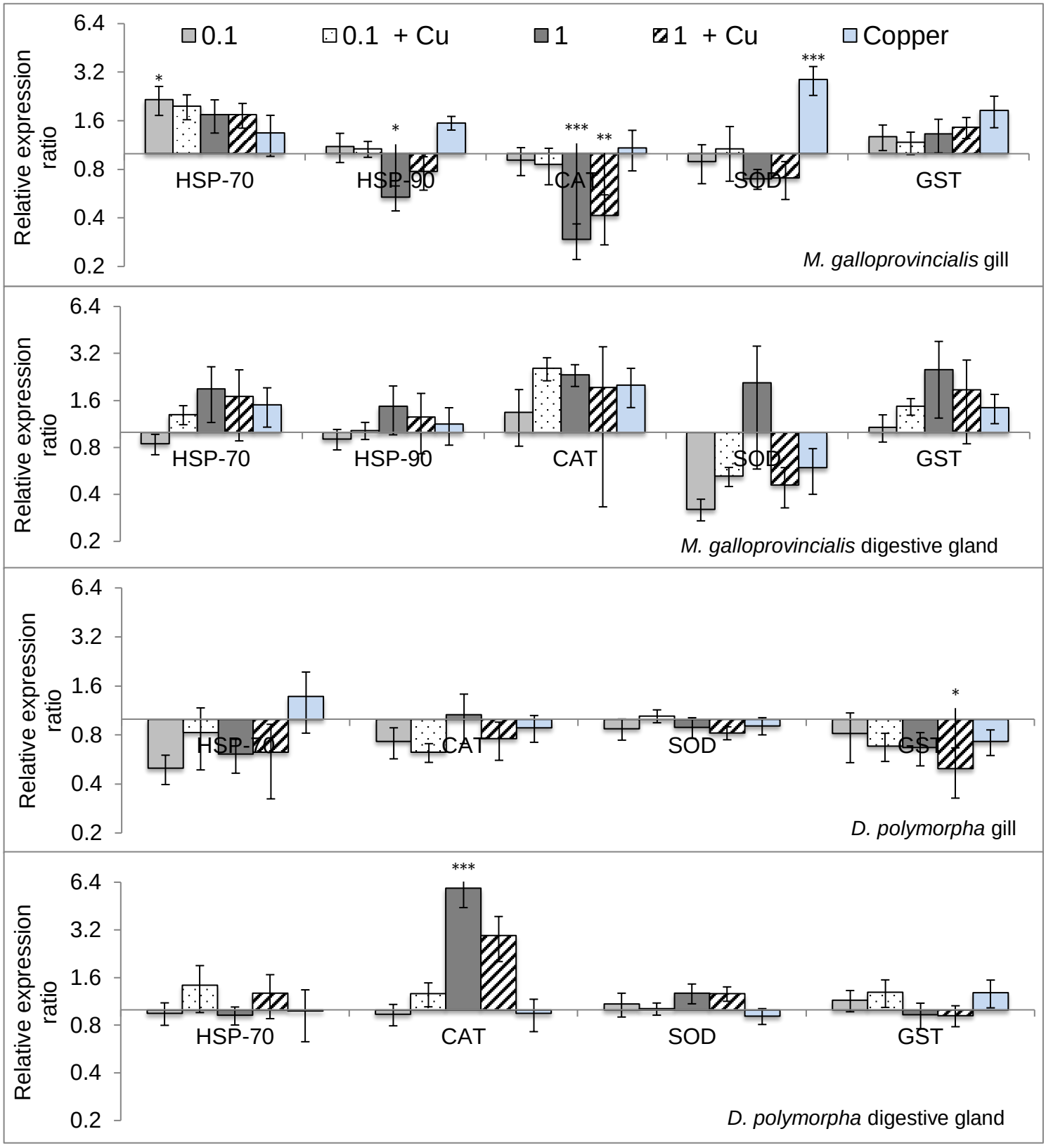


**Figure 3.** Relative expression ratios (RER) of key genes in *M. galloprovincialis* and *D. polymorpha* gill and digestive gland cells following a 10-day exposure to $^{32}$P and Cu, alone and in combination. Data are normalised for reference genes (ef1, actin) and controls. Error bars indicate the 95% confidence intervals. Asterisks (*, ** or ***) are indicative of significant differences ($p < 0.05, 0.01, 0.001$) from the corresponding control. $n = 9$. Copper = positive control.

### 3.3 Multivariate analysis of biomarker reactions

Analysis of the potential correlations within the biomarker data indicated that most of the biological parameters were not strongly correlated (Fig. 2a and 2b, A & C), with several exceptions (primarily comet [measure of DNA damage] and γ-H2AX assays, across all tissues, $p < 0.05$). PCA and MDS (MDS plots not shown) combined with cluster analysis showed that the experimental treatments were clearly distinct from the control groups (Fig. 2a and b, B & D). The percentage of variance explained by PC1 and PC2 were as follows: DP digestive gland, 32.6% and 21.4%; DP gill, 33.2% and 24.3%; MG digestive gland, 47.5% and 27.1% and MG gill, 32.9% and 28.4%. ANOSIM analysis gave global significances of $p < 0.001$ for both tissues and species. In DP gill tissue, pairwise analysis showed that all treatments were significantly different from one another ($p < 0.05$), digestive gland tissue of this species showed similarly distinct differences between pairs of treatments except for 0.1 mGy $d^{-1}$ and 0.1 mGy $d^{-1}$ + Cu. MG gill tissue showed clear differences between pairs of treatments except for 0.1 mGy $d^{-1}$ and 1 mGy $d^{-1}$, and 1 mGy $d^{-1}$ and 1 mGy $d^{-1}$ + Cu. However, the digestive gland tissue of MG showed greater overlap in treatments, with no significant differences between 0.1 mGy $d^{-1}$ and 1 mGy $d^{-1}$ + Cu, 0.1 mGy $d^{-1}$ and positive Cu Control, 1 mGy $d^{-1}$ and 1 mGy $d^{-1}$ + Cu, and 1 mGy $d^{-1}$ + Cu and positive Cu control. The PCA results for this tissue showed that the experimental treatments were more strongly grouped together than in any other tissues (Fig. 2B).

As already stated, most biomarkers were not correlated with each other, and the BIO-ENV routine for various combinations of biomarkers indicated that there were no influential biomarkers among the various combinations capable

of capturing the full PCA biomarker response pattern. Principal component (PCA) coupled with hierarchical cluster analysis and ANOSIM results for all treatments showed that both $^{32}P$ and Cu had a detrimental effect on the genetic integrity and oxidative stress status in the four tissues tested (Figs. 2a and b, A - D).

## 4 Discussions

This novel study is the first to explore the interactive effects of $^{32}P$ and Cu on two aquatic bivalve species using a multi-biomarker approach. Qualities of radionuclides and speciation of metals (i.e. physical and chemical properties) in aquatic systems has a large influence on bioavailability and subsequent toxicity (Gunten and Beneš 1995; Richards et al. 2011). It is well documented that Cu, focusing on the toxic ionic form ($Cu^{2+}$) forms complexes with natural organic matter, decreasing bioavailability and at lower salinities (i.e. freshwater) becoming more abundant (Grosell et al. 2007). Despite this, little is known on the possible interactions between Cu and lesser studied radionuclides such as $^{32}P$ and the combined influence of water parameters (i.e. pH, salinity, dissolved organic carbon [DOC], alkalinity). Due to time and logistical constraints certain parameters (e.g., DOC) were not determined during this experiment. It is possible that differing water chemistry (i.e. salt and freshwater), along with chemical interactions between stressors could have affected the noted biological effects in each species.

### 4.1 Biomarker interactions

PCA can be used to integrate information from multiple biological parameters. Our study demonstrates that PCA can aid interpretation of multi-biomarker responses to combined environmental stressors (i.e. IR and metals), by reducing multi-dimensionality into a less complex two-dimensional representation (Chatfield and Collins 1980; Allen and Moore 2004; Moore et al. 2006). However, PCA and cluster analysis are limited in the inability to integrate biomarkers in a more functional manner, wherein, the endpoints (i.e. DNA damage, MN formation) adopted in this study are not necessarily reflective of 'overall health status'. It is likely that such biomarkers indicate whether a contaminant-induced response has taken place, or that health status within a narrow range has been altered, but they do not generally indicate an individual's health status for the whole range from healthy to permanent damage (Depledge et al. 1993; Köhler et al. 2002; Moore et al. 2004; Moore et al. 2006). Despite this, subsequent investigations could build on the information provided in order to develop such models in the future. Furthermore, this study is certainly useful in establishing relationships between stressor and response, PCA coupled with hierarchical cluster analysis and ANOSIM results for all treatments showed that both $^{32}$P and Cu had deleterious effects on genetic integrity and oxidative stress status in species and tissues tested (Fig. 2a and b, A - D).

### 4.2 $^{32}$P induced genotoxic response in gill and digestive gland cells

The majority of IR-induced toxicity studies have focused on single radionuclide exposures. DNA damage as a biomarker has been noted at dose rates ranging from 0.8-41666 µGy $h^{-1}$ in a wide range of biota, including marine bivalves (*Mytilus* spp., *C. gigas*, *P. perna*, *C. fluminea*, *P. malabarica* & *M. casta;* Jha et

al. 2005; Jha et al. 2006; Godoy et al. 2008; Farcy et al. 2011; AlAmri et al. 2012; Kumar et al. 2014). PCA and MDS, combined with cluster analysis demonstrate a clear distinction between controls and experimental groups, across both species, tissue and biomarker (Fig. 2a and b, B & D). In keeping with previous work (Vernon et al. 2020), no significant change in DNA damage was noted at lower $^{32}P$ doses of 0.10 mGy $d^{-1}$ (Excluding DP digestive gland, present study). However, where previous findings demonstrated significant increased % Tail DNA in both species and cell types at 1 mGy $d^{-1}$, only MG digestive gland was significantly increased. The present study had greater baseline levels of DNA damage of ~5-11%, compared to <5% in some previous literature, this along with individual differences may account for such variation.

Exposure of the mussels to $^{32}P$ alone caused no significant increase in % Tail DNA (Excluding DP [0.1] and MG digestive gland [1 mGy $d^{-1}$]), but when combined with Cu, % Tail DNA was statistically greater than controls in MG gill and DP digestive gland at 0.10 mGy $d^{-1}$, and all species and tissues at 1 mGy $d^{-1}$. Cu in isolation, at environmentally relevant concentrations (18 µg $L^{-1}$) has been found to have no significant effect on % Tail DNA in MG or DP gill cells (Vernon and Jha 2019), when in combination with $^{32}P$ there appears to be an additive effect on mussels. Whilst not significantly so the addition of Cu to IR exposures also increased γ-H2AX foci induction, across both species, tissue and dose rate (excluding MG digestive gland). This apparent additive effect of Cu on the genotoxicity of $^{32}P$ on marine and freshwater mussels is the first reported. Cu-contaminant induced effects have been noted in previous literature. In *M. edulis,* Cu-induced (0.1 µM) damage to DNA and lipids was noted as significantly greater under low pH conditions (reflective of ocean

acidification), in comparison to controls (Lewis et al. 2016). Similarly, combined Cu (10 µg $L^{-1}$) and IR ($^{60}Co$, 70 mGy) exposure was found to induce significantly depleted glutathione compared to exposure to Cu alone, in presmolt salmon, *S. salar* (Heier et al. 2013).

Aquatic biota are continuously exposed to both endogenously and environmentally generated contaminants, giving cause for the development of highly effective biochemical mechanisms that afford the ability to protect and defend on multiple biological levels. H2AX is quickly phosphorylated to form γ-H2AX, a crucial factor in DSB repair response and therefore a relevant, useful technique in radiation biology, where the MoA (mechanism of action) of $^{32}P$ in particular is mediated by induction of DNA DSBs (Kuo and Yang 2008; Cheng et al. 2015). Pereira et al. (2011), Urushihara et al. (2012), Pereira et al. (2014 and Sayed et al. (2017) have successfully utilised this technique to assess DDR in fish (*O. latipes* and *D. rerio*) following acute exposures to $^{137}Cs$, but to our knowledge, is yet to be fully utilised in mussels. The γ-H2AX assay is further validated when utilised alongside classical DNA damage techniques, such as comet and MN assays, relationships have been demonstrated between such biomarkers (Pereira et al. 2011; Sayed et al. 2017; Dallas et al., 2013, 2018). In our study, γ-H2AX was strongly correlated with DNA damage across the tissues ($p < 0.05$), correlation was not noted between other molecular or genetic biomarkers (Fig. 2a and 2b, A, C). The greatest induction of γ-H2AX foci was noted in MG digestive gland, specifically at 1 mGy $d^{-1}$ (~ 19 foci/cell), followed by the 1 mGy $d^{-1}$ + Cu, positive Cu control, 0.1 mGy $d^{-1}$+ Cu and 0.1 mGy $d^{-1}$ treatments. At significantly higher dose rates of 10 mGy $d^{-1}$ ($^{137}Cs$), less than 5 foci were present per zebrafish ZF4 cell (Pereira et al. 2011). γ-H2AX foci

occurs rapidly post-irradiation (30 min – 2 h) and decrease in number over time, as both studies measured damage immediately (~10-40 min) post exposure, varying radiosensitivity may be a result of species, radionuclide source or associated MoA (Sedelnikova et al. 2003; Ivashkevich et al. 2011; Ivashkevich et al. 2012). Trends in γ-H2AX foci induction in MG digestive gland followed DNA damage, but not MN formation, where MN/1000 cells remained uniform respective of experimental treatment. The presence of MN is generally regarded as more long-lasting damage, suggesting that the induced DSBs were easily repairable. This was further noted in MG gill cells.

In line with previous studies (Vernon et al. 2020), a greater degree of MN formation is noted in DP gill and digestive gland cells, relative to associated MG tissue, across all treatments, suggesting a significant impact on DNA integrity. Unexpectedly Cu has a weak antagonistic interaction with $^{32}P$ in terms of MN induction, where MN/1000 cells decrease in DP digestive gland (both $^{32}P$ concentrations), although not significantly so. It is possible that Cu-specific repair mechanisms, as a by-product buffer against the more permanent effects induced by IR as an isolated threat (Yu et al. 2012).

As described in a previous study (Vernon et al. 2020), $^{32}P$ concentrates differentially in gill and digestive gland tissues, with the latter accumulating 87 % (MG) and 44% (DP) of $^{32}P$ (proportion of whole body $^{32}P$ concentration) at 10 mGy $d^{-1}$. Similarly, Cu uptake varies between tissue and species (Cai and Wang 2019; Vernon and Jha 2019). In turn, there are clear differences in tissue-specific dose rate (Table 1) and subsequent genotoxic response to IR and metals, highlighting the importance of a multiple-tissue approach in ecotoxicological studies. Overall, the addition of Cu arguably has the lowest

impact on $^{32}$P-induced damage in MG digestive gland, where MG gill tissue shows far less variation between treatments (excluding % Tail DNA, 0.1 and 0.1 + Cu). In keeping with previous findings, in terms of longer-term damage, high MN frequency in DP cells suggests a more long-lasting response in freshwater mussels, in relation to MG.

### 4.3 Transcriptional expression of key genes

In our study, transcriptional expression of key genes showed little variation between differing treatments. The findings are in agreement with previous work (Vernon et al. 2020). Excluding significant upregulation of *sod* in MG gill, the Cu positive control (56 µg $L^{-1}$) caused no variation in gene expression. This is in contrast to work by Xu et al. (2018) where exposure to much lower Cu concentrations (2 and 8 µg $L^{-1}$) significantly induced the expressions of stress responsive genes (*hsp70, hsp90, mt-10*) in *Mytilus coruscus* (haemocytes), particularly 12 d post exposure. Furthermore, significantly altered transcriptional profiles in fish (*D. rerio* and *S. salar*) have been noted following IR exposure ($^{137}$Cs or $^{60}$Co). Common MoAs associated with low-dose gamma radiation included the induction of oxidative stress and DNA damage (Jaafar et al. 2013; Freeman et al. 2014; Song et al. 2014; Song et al. 2016). At a molecular level, lack of significant variation between response may be an indication of disparity in toxicity MoA, or different sensitivities between the end points (Devos et al. 2015). Noted genomic damage may result from the direction interaction of $^{32}$P with DNA, through the release of high-energy beta particles or isotopic decay, and to a lesser extent via the generation of ROS.

Due to both logistical constraints and lack of readily available gene sequences for MG and DP, we were only able to assess five key genes in this study. This provides limited information pertaining to molecular mechanistic responses to IR and Cu. Furthermore, while identification of transcriptome variation is undoubtedly important and increasingly popular within radiobiological research, limited information can be acquired by studying genes in isolation. If this work were taken further, it would be logical to use microarrays, RNAseq and proteomics analyses (Banni et al., 2017; Dallas et al., 2018; Barranger et al., 2019 a,b) and at multiple, earlier time points and at different environmentally relevant temperatures as performed in previous studies in our laboratory conditions order to dissect out the mechanisms of toxicity (Dallas et al., 2013, 2016, 2018). Identifying known and potentially novel molecular targets (genes and proteins) involved, as well as activated signalling pathways in a range of biological systems under multi-stress exposure scenarios may be the first step in predicting potential larger scale impacts. These could eventually include detrimental effects at high levels of biological organisation, such as community or population level (Maria et al. 2013; Gomes et al. 2014).

Along with other hydrological characteristics (e.g. pH, salinity, dissolved oxygen) which show temporal and seasonal variabilities in the natural environment, temperature is considered to be an important factor which is known to influence many biological responses (e.g. expression of genes, induction of DNA damage) in aquatic organisms (Jha, 2008; Dallas and Jha, 2015). It can also influence cell division and therefore cell-cycle dependent phenomenon such as induction of micronuclei (MN) in tissue and species-specific manner. Furthermore, given that thermal discharge from nuclear power plants is considered to be an important environmental issue along with potential sea surface temperature rise by up to 3.5° C in the next 100 years (IPCC, 2007), determining the interaction

of elevated temperature with radionuclide and other environmental stressors (e.g. metals) could be considered highly relevant. Previous study from our laboratory has elaborated potential interactive impact of temperature and an environmentally relevant radionuclide (i.e. tritium) on these parameters in marine mussels (Dallas et al., 2016). In line with most of the studies, carried out by us and other workers, the experiments in this study were carried out at 15°C, which is considered to be environmentally relevant for these species. In future, it would be helpful to elucidate the impact of temperature and other water quality parameters while comparing the relative sensitivities of different species following exposure to multiple stressors.

## 5 Conclusions

In conclusion, the results of this integrated, multi-biomarker study represent the most extensive data to date obtained on the combined effects of IR and metals (Cu) in two environmentally relevant bivalve species. The results indicate that (a) genotoxic response was reflective of exposure, where Cu had an overall additive effect on $^{32}P$-induced damage across several (but not all) species, cell types and dose rates, (b) selected genes were generally unaltered in terms of transcriptional response to contaminants, independent of species and (c) in exclusion of comet and γ-H2AX assays biological parameters were not strongly correlated. Whilst it is difficult to extrapolate such findings to exposures in realistic environmental conditions, these data contribute to the limited amount of information available in the literature on the possible mechanisms involved in multi-stressor (i.e. IR and metals) induced biological responses. The study highlights the importance of investigating the interactive effects of pollutants on ecologically relevant species.

## Disclosure statement

The authors declare no conflict of interest.


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## Notes on contributors


***Dr Emily L. Vernon*** is a post-doctoral research fellow working in the areas of ecotoxicology and environmental radioactivity in the School of Biological and Marine Sciences, University of Plymouth, UK.

***Professor Michael N. Moore*** is a Consultant Environmental Toxicologist, Cell Pathologist and Systems Biologist. He is an Honorary Professor at the European Centre for Environment and Human Health (ECEHH), University of Exeter Medical School, Truro; and at the School of Biological & Marine Sciences, University of Plymouth; and also an Emeritus PML Fellow at Plymouth Marine Laboratory.

***Dr Tim P. Bean,*** is a Career Track Fellow at the Roslin Institute, University of Edinburgh. His research group uses molecular techniques to study aspects of fundamental bivalve biology which have direct implications for aquaculture industry and environmental policy.

***Professor Awadhesh N. Jha***, is a Professor of Genetic Toxicology and Ecotoxicoloy in the School of Biological and Marine Sciences, University of Plymouth, UK. His research interest involves elucidation of interactions of environmental stressors including anthropogenic chemicals and radiations with the genomes and its potential impact on the health of humans and wild species.

**ORCID**

Awadhesh N. Jha http://orcid.org/0000-0001-9660-4308

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**Table Captions and Figure legends**

**Table 1.** Table to show the expected dose rates in mGy d-1 and the average dose rate achieved in M. galloprovincialis and D. polmorpha whole-body, digestive gland and gill tissue (mGy d-1). . Values in bold are those above the ERICA tool screening value of 0.24 mGy d-1.

**Table 2.** Water quality parameters (pH, temperature, salinity and dissolved oxygen [DO]), copper concentrations in water (µg $L^{-1}$) and $^{32}P$ concentrations in water (Bq $L^{-1}$), for both species. Data is presented as mean ± standard deviation. *n* = 3.

**Table 3.** Gene and primers used for *M. galloprovincialis* and *D. polymorpha* in RT-PCR.

**Figure 1.** Genotoxic effects and subsequent repair in *M. galloprovincialis* and *D. polymorpha* gill and digestive gland cells following a 10 day exposure to $^{32}P$ and Cu, alone and in combination. Asterisks (*, ** or ***) are indicative of significant differences ($p < 0.05, 0.01, 0.001$) from the corresponding control. Letters are indicative of significant differences ($p < 0.05$) between species tissue (i.e. MG gill tissue). SD is standard deviation of mean data. *n = 9.* Copper = positive control.

**Figure 2a**. Multivariate analysis (PRIMER 6.1.5) between treatments for the integrated biomarker data showing scatter plots (A, C), principal component (PCA) and cluster analysis of the biomarker data. Vectors indicate the directionality of specific biomarkers. (A, B) *M. galloprovincialis* digestive gland and (C, D) gill tissue. Asterisks (*) are indicative of significant differences ($p < 0.05$). PC1 captured (B) 47.5 % and (D) 32.9 % of the variation.

**Figure 2b**. Multivariate analysis (PRIMER 6.1.5) between treatments for the integrated biomarker data showing scatter plots (A, C), principal component (PCA) and cluster analysis of the biomarker data. Vectors indicate the directionality of specific biomarkers. (A, B) *D. polymorpha* digestive gland and (C, D) gill tissue. Asterisks (*) are indicative of significant differences ($p < 0.05$). PC1 captured (B) 32.6 % and (D) 33.2 % of the variation.

**Figure 3.** Relative expression ratios (RER) of key genes in *M. galloprovincialis* and *D. polymorpha* gill and digestive gland cells following a 10-day exposure to $^{32}P$ and Cu, alone and in combination. Data are normalised for reference genes (ef1, actin) and controls. Error bars indicate the 95% confidence intervals. Asterisks (*, ** or ***) are indicative of significant differences ($p < 0.05, 0.01, 0.001$) from the corresponding control. *n = 9.* Copper = positive control.